Saskatchewan

A D F

Agriculture Development Fund

FINAL REPORT

IDENTIFICATION OF FLEA BEETLE RESISTANT ARABIDOPSIS GERMPLASM

19970279

Funded by
The Agriculture Development Fund
March 1999

Prepared by: Agriculture and Agri-Food Canada

SAF
Saskatchewan Agriculture & Food
Adding value to agriculture...through research and development

100-05112/

IDENTIFICATION OF FLEA BEETLE RESISTANT *ARABIDOPSIS* GERMPLASM
ADF PROJECT NO. 97000279

MAY 20 1999

FINAL REPORT, MARCH 1999
Prepared by Dr. Rebecca H. Hallett

TECHNICAL REPORT

Objectives:
To identify sources of flea beetle resistance genes by screening ecovars and mutant lines of *Arabidopsis thaliana*. The long-term goal is to map insect resistance and susceptibility in the *Arabidopsis* and canola genomes and to incorporate resistance genes into canola for the development of flea beetle resistant cultivars.

Project Activities:

Preliminary work.
Preliminary work was undertaken in 1997 (prior to this ADF Grant) in the laboratories of Drs. J.J. Soroka and M.Y. Gruber to develop a means of assaying resistance of *A. thaliana* to crucifer flea beetles (see description in "Construction of screening arenas" below).

Plant developmental times.
Seed stocks of 66 wild ecotypes and mutant lines of *A. thaliana* were increased in a greenhouse during the winter and spring of 1998.

Developmental rates of these lines were determined in May 1998. Seeds were surface sterilized and placed in petri dishes containing agar (Figure 1). Plates were kept in the dark at 4°C for 3 days, then incubated at 22°C in the light (16 hr photoperiod, 70-120 $\mu E/m^2/sec$) (Figure 2). Plates were checked daily for time to germination, emergence and expansion of first pair of true leaves and emergence and expansion of second pair of true leaves.

Approximately 34 additional lines of *A. thaliana* were purchased in August 1998 and seed increases of these lines were made in the greenhouse from September 1998 to March 1999. Developmental times of all these lines were determined during the winter of 1999.

***Arabidopsis* screening arenas.** *A. thaliana* seedlings were transplanted at the first true leaf stage (12-15 days after plating) into the centre 64 wells of a 96-well microtitre plate containing agar (Figure 3). Microtitre plates were placed in a sealed plastic container and incubated for 2 days prior to conducting the flea beetle assay (Figure 2). The assay arena consists of a 96-well microtitre plate with a modified top to contain flea beetles and allow ventilation (Figure 4). A cork hole in the top allows introduction of flea beetles to the arena. Eight lines of *A. thaliana*, arranged in an 8x8 Latin Square design, can be screened simultaneously. Screening arenas were constructed in April 1998.

Flea beetle resistance assays. Preliminary experiments to develop an assay for screening *A. thaliana* lines for flea beetle resistance were conducted in 1997. In June 1998, four experiments were conducted to test the reliability of the assay procedure. All experiments contained the same check ecotype and were replicated four times. The check ecotype, Columbia, was selected because it is the source plant for many of the mutants of *A. thaliana* that have been developed and that were screened in this study. In Exps. 1-3, twenty wild ecotypes and one mutant line were screened. In Exp.4, the most promising lines from preliminary experiments in 1997 and one additional mutant line were re-assessed.

A visual guide for rating damage in 10% leaf area increments was developed in August 1999 and utilized in Exps. 5 & 6 in order to standardize ratings between different people. Experiments 5 & 6 were conducted in September and October 1998.

Significant differences in the level of flea beetle herbivory were found among *A. thaliana* lines in all experiments, demonstrating the ability of this assay to detect quantitative differences in damage levels between lines.

Resistance to flea beetles differed with seedling tissue type (i.e. cotyledons or true leaves) within lines. When looking for sources of resistance genes that may be differentially expressed at different developmental stages of the plant, separate examination of damage levels to different tissue types is important.

In this series of experiments, no highly resistant lines were identified. However, significant variation in damage levels was found. These results indicate that expression of resistance in the *A. thaliana* genome is plastic and that further screening may identify highly resistant lines. This work will be continued Spring - Fall 1999 in order to assay lines that were increased in Winter 1999. It is hoped that sources of flea beetle resistance genes can still be identified by screening ecovars and mutant lines of *Arabidopsis thaliana*. The long-term goal is to map insect resistance and susceptibility in the *Arabidopsis* and canola genomes and to incorporate resistance genes into canola for the development of flea beetle resistant cultivars.

Flea Beetle Maintenance Colonies. Preliminary studies conducted by R.H. Hallett, indicated that flea beetles may be kept in a semi-active state throughout the winter, if maintained on canola leaves at 10°C. In order to determine whether beetles kept in this semi-active state could be removed throughout the winter and used in feeding assays, maintenance colonies of beetles were established in the fall of 1998.

Flea beetles collected in September 1998 were held under long (18 hr) or short (6 hr) day conditions at 10°C. Three different sizes of container were tested: 500 mL (250 beetles per container), 2 L (1,000 beetles per container) or 4 L (2,000 beetles per container) (Figure 5). Containers were inverted and fitted with mesh-covered holes for ventilation. Beetles were provided with sufficient fresh cabbage leaf pieces for the number of beetles and fresh food was provided each week. Beetles also had access to a sucrose solution provided through a wick inserted into the side of the container. Unfortunately, most beetles (70-95%) died within 6 weeks of establishing the maintenance containers. A feeding assay was conducted with beetles from a 4 L container treatment on November 3. It was determined, by comparing the data from this

experiment with data obtained in similar experiments conducted over the summer with freshly collected field beetles, that feeding patterns of stored and freshly collected beetles are not the same and hence results obtained from assays with stored beetles should be used with caution.

EQUIPMENT

No expenditures have been made to date on equipment purchases.

PROJECT DEVELOPED MATERIALS

A poster entitled "*Arabidopsis thaliana* as a potential source of flea beetle resistance genes" by Rebecca H. Hallett, Heather Ray, Margaret Y. Gruber, and Juliana J. Soroka was presented by Rebecca Hallett at the 34th Annual Meeting of the Canadian Botanical Association, 27 June - 1 July 1998, Saskatoon, SK. A copy of the poster is attached to this document.

A poster entitled "A method for assessing crucifer flea beetle resistance in *Arabidopsis thaliana*" by Rebecca H. Hallett, Heather Ray, Margaret Y. Gruber, and Juliana J. Soroka was presented by Rebecca Hallett at the Annual Meeting of the Entomological Society of Canada to be held in Quebec City, October 31 - November 4, 1998. A copy of the poster is attached to this document.

A paper entitled "Assessing crucifer flea beetle resistance in *Arabidopsis thaliana*" by Rebecca H. Hallett, Heather Ray, Margaret Y. Gruber, and Juliana J. Soroka is in preparation and will be submitted for publication in Fall 1999.

PROJECT PHOTOS

Relevant photos are included as Figures 1-5.

ACKNOWLEDGEMENT

Support by the Saskatchewan Department of Agriculture & Food was acknowledged on the posters presented at the Canadian Botanical Association Meeting and the Entomological Society of Canada Meeting (see attached), and will be acknowledged in all future publications and presentations.

Figure 1. Petri dishes containing agar for germination of *Arabidopsis* seedlings. This set-up was used for both developmental time studies and resistance bioassays.

Figure. 2. Germination plates and 96-well microtitre plates being incubated in controlled growth room under at 22°C in the light (16 hr photoperiod, 70-120 μE/m²/sec)

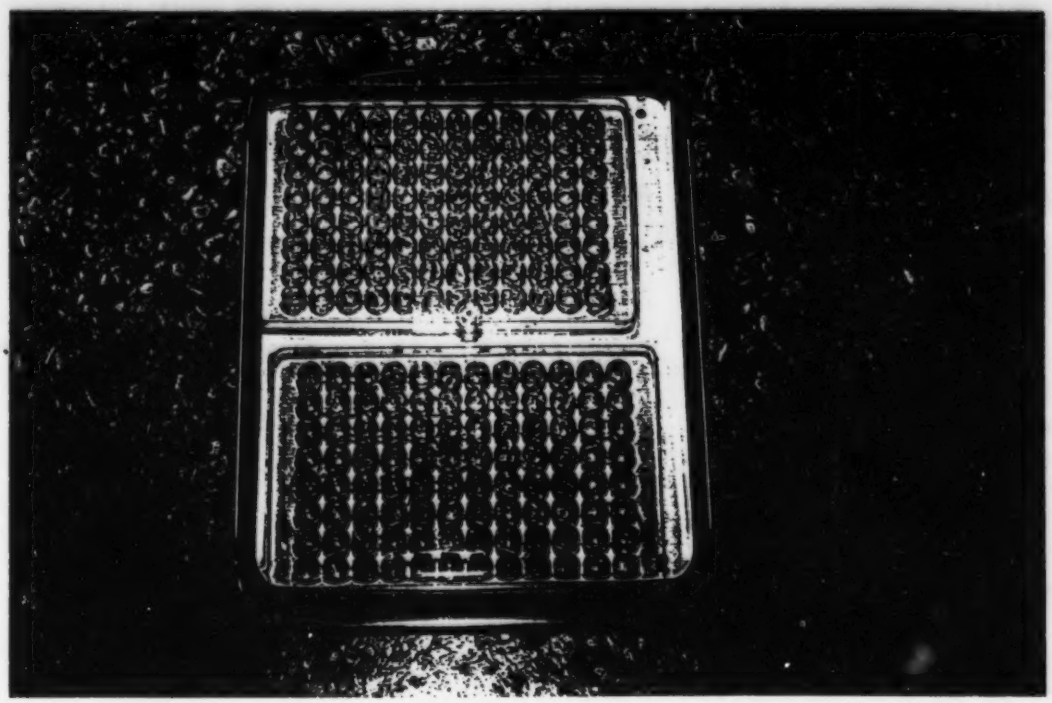


Figure 3. *Arabidopsis* seedlings transplanted into an 8x8 Latin Square array in centre 64 wells of 96 well microtitre plate.

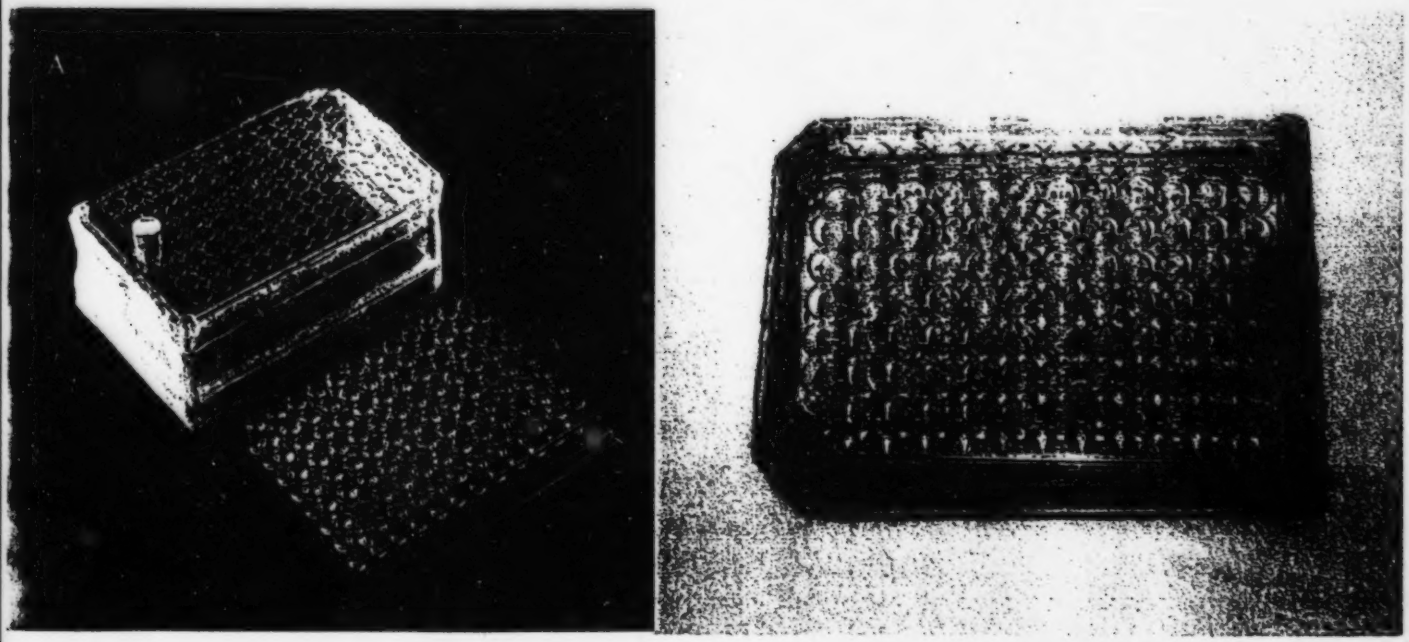


Figure 4. Arena for assaying resistance of *Arabidopsis* lines to crucifer flea beetle feeding. **Left.** Unassembled arena showing 96 well microtitre plate and screened lid. **Right.** Assembled arena containing 50 flea beetles.

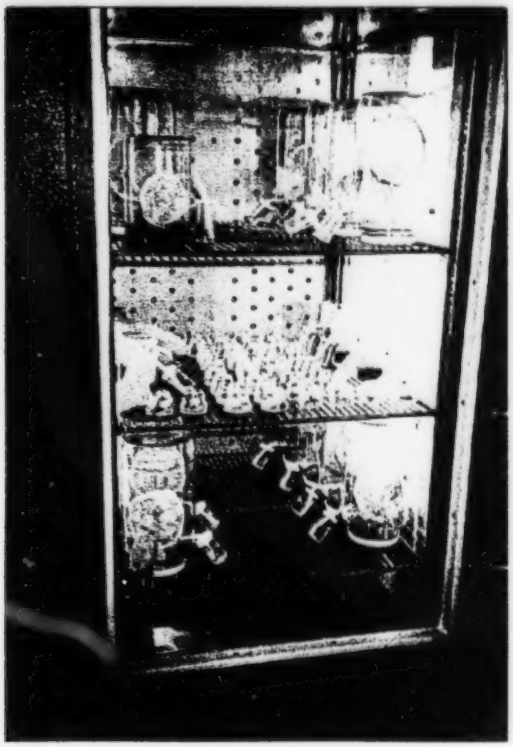


Figure 5. Controlled environment growth chamber for flea beetle maintenance colonies. 4 L containers on top and bottom shelves, with white lids; 2 L containers on top shelf with red lids; 500 mL containers on middle shelf with red shelf. Vials of sucrose solution provided moisture and food energy to flea beetles in addition to fresh cabbage leaves provided weekly.

CRUCIFER FLEA BEETLE RESISTANCE GENES

Rebecca H. Hallett, Heather Ray, Margaret Y. Gruber & Juliana J. Soroka
Saskatoon Research Centre, Agriculture & Agri-Food Canada.

INTRODUCTION

Crucifer flea beetles, *Phyllotreta* spp. (Coleoptera: Chrysomelidae), are serious pests of canola. Adult beetles feed on canola seedlings in the spring (Fig. 1), causing reduced plant densities, reduced growth, delayed maturity and lower yields. Despite pesticide measures, reductions in yield cause revenue losses of $130-160 million per year.

The development and adoption of resistant canola cultivars would reduce control costs and losses to flea beetles, resulting in significant savings to the producer and alleviation of the negative environmental effects of pesticide use. Important sources of resistance genes are cruciferous plants related to canola, but not fed upon by flea beetles. This type of resistance is durable and can be identified and isolated very quickly from the weedy species *Arabidopsis* compared to other crucifers. *Arabidopsis* has a rapid growth cycle, simple genome, a close relationship to canola, and a large number of unique lines available for screening.

We developed a method for assaying the resistance of wild and mutant lines of *Arabidopsis thaliana* to crucifer flea beetles.

Fig. 1. Crucifer flea beetles feeding on canola.

ASSAY METHODOLOGY

An assay for screening *Arabidopsis* lines for flea beetle resistance was developed in 1997. In 1998, four experiments were conducted to test the reliability of the assay procedure (Fig. 2). All experiments contained the same check ecotype and were replicated four times. In Expts. 1- 3, 20 wild ecotypes and one mutant line were screened. In Exp. 4, the most promising lines from preliminary experiments in 1997 and one additional mutant line were re-assessed.

The assay arena consists of a 96 well microtitre plate with a modified top to contain flea beetles and allow ventilation (Fig. 3). A cork hole on the top allows introduction of flea beetles to the arena. Eight lines of *Arabidopsis*, arranged in an 8x8 Latin Square design, are screened simultaneously.

Arabidopsis seeds were surface sterilized by soaking for 2 min. in 70% ethanol, followed by 3 min. in 10% bleach and 3 rinses with sterile distilled water. Sterilized seeds were resuspended in 0.1% agar and pipetted with wide bore tips onto deep petri dishes (100x20mm) containing 0.8% agar supplemented with 2.2 g/L Murashige Minimal Organics Medium without sucrose (Gibco BRL). Plates were kept in the dark at 4°C for 3 days, then incubated at 22° C in the light (16 hr photoperiod, 70-120 $\mu E/m^2/sec$).

At the first true leaf stage (12-15 days after plating), seedlings were transplanted in an 8x8 array into 96 well microtitre plates containing 1.5% agar with the same salt concentration as above. The open microtitre arenas were placed in sealed clear plastic boxes to prevent desiccation and were incubated for two more days. Fifty crucifer flea beetles, starved for 24 hrs, were then introduced into assembled arenas using an aspirator. Bioassays were conducted in a controlled environment growth room (24° C day, 18° C night, 16 hr photoperiod). Beetles were removed after check plants experienced approx. 70% damage (about 15-18 hrs).

After removal of flea beetles, all seedlings were observed *in situ* under a dissecting microscope and two cotyledons and two true leaves per plant were rated using a 6 point scale (0, 10, 30, 60, 90, 100% damage). The mean of these values was used as the percent damage rating for each plant. Percent damage ratings were analyzed by ANOVA and means separations were calculated using Duncan's multiple range test.

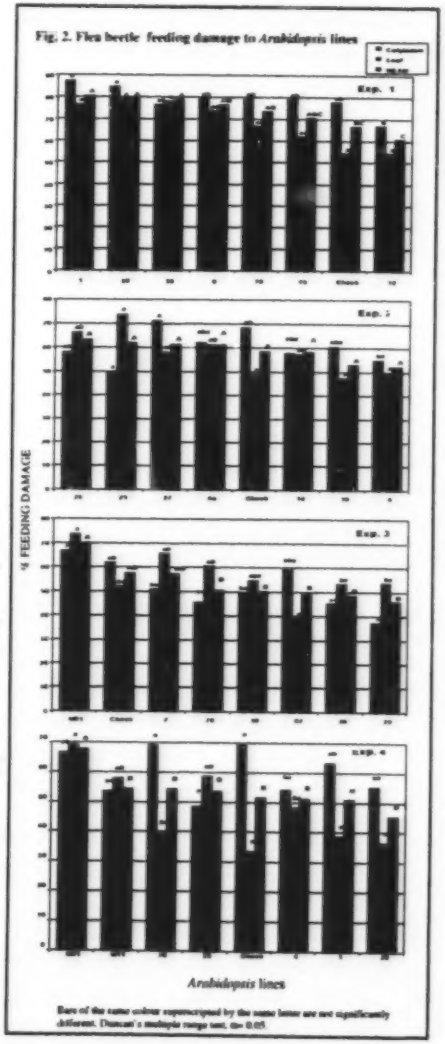


Fig. 2. Flea beetle feeding damage to Arabidopsis lines
Cotyledon
Leaf
Mean
Exp. 1
Exp. 2
Exp. 3
Exp. 4
% FEEDING DAMAGE
Arabidopsis lines
Bars of the same colour superscripted by the same letter are not significantly different. Duncan's multiple range test, α= 0.05.

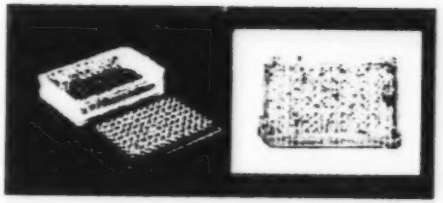


Fig. 3. Arena for assaying crucifer flea beetle resistance in *Arabidopsis*.

RESULTS & DISCUSSION

Significant differences in the level of flea beetle herbivory were found among *Arabidopsis* lines in all four experiments (Fig. 2). Thus, this assay is able to detect quantitative differences in damage levels between lines.

While significant differences were found between replicates in many comparisons, these were related to differences in light intensity in the assay room. The Latin square design was selected to minimize these differences within an arena. In no case was the replicate x line interaction significant. These results confirm the validity of the bioassay in determining the relative susceptibility or resistance of *Arabidopsis* lines to flea beetle damage.

Resistance to flea beetles differed with seedling growth stage within lines (eg. Exp. 4, Check). In Exp. 2, significant differences were found between lines for cotyledon damage (green bars) and for first true leaf damage (blue bars). However, these differences were not evident in pooled analysis of the mean damage levels per seedling (red bars). When looking for sources of resistance genes that may be differentially expressed at different developmental stages of the plant, separate examination of damage levels to different tissue types is important.

Physiological condition of flea beetles used in these assays was also very important. Field-collected flea beetles that were kept in growth room conditions for more than one week fed less than did freshly collected beetles. For this reason, we used only beetles that were collected less than one week earlier. There may also be differences in feeding preferences between reproductively mature adults present in the spring and non-reproductive adults present in the late summer. Thus, in future tests all lines of *Arabidopsis* will be screened against both spring and summer flea beetles.

In this series of experiments, no highly resistant lines were identified. However, significant variation in damage levels was found. These results indicate that expression of flea beetle resistance in the *Arabidopsis* genome is plastic and that further screening may identify highly resistant lines. Ultimately, our goal is to map insect resistance and susceptibility in the *Arabidopsis* and canola genomes, and to incorporate resistance genes into canola for the development of flea beetle resistant cultivars.

This project was supported in part by the Saskatchewan Department of Agriculture & Food.

A METHOD FOR ASSESSING CRUCIFER FLEA BEETLE RESISTANCE IN *ARABIDOPSIS THALIANA*

Rebecca H. Hallett*, Heather Ray, Margaret Y. Gruber & Juliana J. Soroka
Saskatoon Research Centre, Agriculture & Agri-Food Canada.
*Current Address: Department of Environmental Biology, University of Guelph

INTRODUCTION

Fig. 1. Crucifer flea beetles feeding on canola.

Crucifer flea beetles, *Phyllotreta* spp. (Coleoptera: Chrysomelidae), are serious pests of canola. Adult beetles feed on canola seedlings in the spring (Fig. 1), causing reduced plant densities, reduced growth, delayed maturity and lower yields. Despite pesticide measures, reductions in yield cause revenue losses of $130-160 million per year.

The development and adoption of resistant canola cultivars would reduce control costs and losses to flea beetles, resulting in significant savings to the producer and alleviation of the negative environmental effects of pesticide use. Important sources of resistance genes are cruciferous plants related to canola, but not fed upon by flea beetles. This type of resistance is durable and can be identified and isolated very quickly from the weedy species *Arabidopsis* compared to other crucifers. *Arabidopsis* has a rapid growth cycle, simple genome, a close relationship to canola, and a large number of unique lines available for screening.

We developed a method for assaying the resistance of wild and mutant lines of *Arabidopsis thaliana* to crucifer flea beetles.

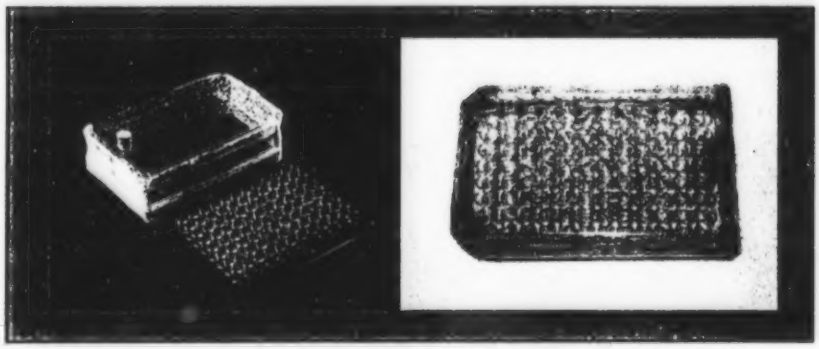


Fig. 2. Arena for assaying crucifer flea beetle resistance in *Arabidopsis thaliana*.

ASSAY METHODOLOGY

An assay for screening *Arabidopsis* lines for flea beetle resistance was developed in 1997. In 1998, eight experiments were conducted to test the reliability of the assay procedure. All experiments contained the Columbia ecotype as a check and were replicated four times.

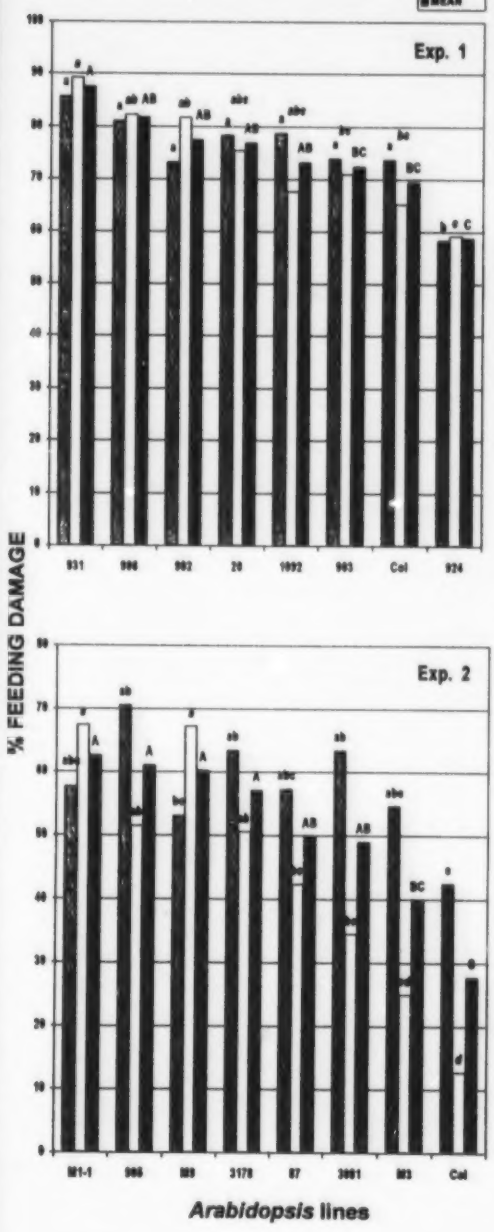
The assay arena consists of a 96 well microtitre plate with a modified top to contain flea beetles and allow ventilation (Fig. 2). A cork hole on the top allows introduction of flea beetles to the arena. Eight lines of *Arabidopsis*, arranged in an 8x8 Latin Square design, are screened simultaneously.

Arabidopsis seeds were surface sterilized by soaking for 2 min. in 70% ethanol, followed by 3 min. in 10% bleach and 3 rinses with sterile distilled water. Sterilized seeds were resuspended in 0.1% agar and pipetted with wide bore tips onto deep petri dishes (100x20mm) containing 0.8% agar supplemented with 2.2 g/L Murashige Minimal Organics Medium without sucrose (Gibco BRL). Plates were kept in the dark at 4°C for 3 days, then incubated at 22° C in the light (16 hr photoperiod, 70-120 $\mu E/m^2/sec$).

At the first true leaf stage (12-15 days after plating), seedlings were transplanted in an 8x8 array into 96 well microtitre plates containing 1.5% agar with the same salt concentration as above. The open microtitre arenas were placed in sealed clear plastic boxes to prevent desiccation and were incubated for two more days. Fifty crucifer flea beetles, starved for 24 hrs, were then introduced into assembled arenas using an aspirator. Bioassays were conducted in a controlled environment growth room (24° C day, 18° C night, 16 hr photoperiod). Beetles were removed after check plants experienced approx. 70% damage (about 15-18 hrs).

After removal of flea beetles, all seedlings were observed *in situ* under a dissecting microscope and two cotyledons and two true leaves per plant were rated using a 0-100% damage scale (10% increments). The mean of these values was used as the percent damage rating for each plant. Percent damage ratings were analysed by ANOVA and means separations were calculated using Duncan's multiple range test. In the experiments presented here (Fig.3), eleven wild ecotypes (20, 87, 902, 903, 905, 906, 924, 931, 1092, 3081, 3178) and three mutant lines (M1-1, M3, M9) were screened for resistance to flea beetles.

Fig. 3. Flea beetle feeding damage to lines of *Arabidopsis thaliana*



Bars of the same colour superscripted by the same letter are not significantly different, Duncan's multiple range test, $\alpha = 0.05$.

RESULTS & DISCUSSION

Significant differences in the level of flea beetle herbivory were found among *Arabidopsis* lines in all four experiments (Fig. 3). Thus, this assay is able to detect quantitative differences in damage levels between lines.

While significant differences were found between replicates in many comparisons, these were related to differences in light intensity in the assay room. The Latin square design was selected to minimize these differences within an arena. In no case was the replicate x line interaction significant. These results confirm the validity of the bioassay in determining the relative susceptibility or resistance of *Arabidopsis* lines to flea beetle damage.

Resistance to flea beetles differed with seedling growth stage within lines (eg. Exp. 2, lines 3081, M3 and Columbia). In Exp. 2, significant differences were found between lines for cotyledon damage (green bars) and for first true leaf damage (yellow bars). When looking for sources of resistance genes that may be differentially expressed at different developmental stages of the plant, separate examination of damage levels to different tissue types is important.

Physiological condition of flea beetles used in these assays was also very important. Field-collected flea beetles that were kept in growth room conditions for more than one week fed less than did freshly collected beetles. For this reason, we used only beetles that were collected less than one week earlier. There may also be differences in feeding preferences between reproductively mature adults present in the spring and non-reproductive adults present in the late summer. Thus, in future tests all lines of *Arabidopsis* will be screened against both spring and summer flea beetles.

In this series of experiments, no highly resistant lines were identified. However, significant variation in damage levels was found. These results indicate that expression of flea beetle resistance in the *Arabidopsis* genome is plastic and that further screening may identify highly resistant lines. Ultimately, our goal is to map insect resistance and susceptibility in the *Arabidopsis* and canola genomes,
and to incorporate resistance genes into canola for the development of flea beetle resistant cultivars.

This project was supported in part by the Saskatchewan Department of Agriculture & Food.